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α-Mangostin, a Dietary Xanthone, Induces Autophagic Cell Death by Activating the AMP-Activated Protein Kinase Pathway in Glioblastoma Cells

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ABSTRACT: This study is the first to investigate the anticancer effects of α-mangostin in human glioblastoma cells. α-Mangostin decreases cell viability by inducing autophagic cell death but not apoptosis. Pretreatment of cells with the autophagy inhibitors 3-methyladenine (3-MA) and bafilomycin or knockdown beclin-1, resulted in the suppression of α-mangostin-mediated cell death. We also found that liver kinase B1 (LKB1)/AMP-activated protein kinase (AMPK) signaling is a critical mediator of α-mangostin-induced inhibition of cell growth. Activation of AMPK induces α-mangostin-mediated phosphorylation of raptor, which subsequently associates with 14−3−3γ and results in the loss of mTORC1 activity. The phosphorylation of both downstream targets of mTORC1, p70 ribosomal protein S6 kinase (p70S6 kinase) and 4E-BP1, is also diminished by activation of AMPK. Furthermore, the inhibition of AMPK expression with shRNAs or an inhibitor of AMPK reduced α-mangostin-induced autophagy and raptor phosphorylation, supporting the theory that activation of AMPK is beneficial to autophagy. A further investigation revealed that α-mangostin also induced autophagic cell death in transplanted glioblastoma in nude mice. Together, these results suggest a critical role for AMPK activation in the α-mangostin-induced autophagy of human glioblastoma cells.

KEYWORDS: α-Mangostin, autophagy, LKB, AMPK, raptor

INTRODUCTION

Glioblastoma multiforme (GBM) is the most common primary brain cancer and one of the most lethal primary malignancies in humans.[1] Glioblastoma patients have a poor prognosis, with a patient survival rate at 5 years after diagnosis of less than 5% and a median survival of only 14.6 months.[1,2] Glioblastoma is highly resistant to radio- and chemotherapy, and there is no effective cure for patients.[2] Effective chemopreventive treatment for brain cancer would have a tremendous impact on brain cancer morbidity and mortality.

Autophagic cell death is an important physiological process of all eukaryotic cells.[3,4] Autophagic cell death is characterized by massive degradation of cellular contents, including portions of the cytoplasm and intracellular organelles, by means of complicated intracellular membrane/vesicle reorganization and lysosomal hydrolysis.[3−5] Autophagic cell death is important for development and stress responses and has also been observed in several human diseases, including neurodegenerative and muscular disorders, as well as resistance to pathogens.[3] Furthermore, similar to apoptosis, autophagic cell death is suppressed in malignant tumors. A number of studies have reported that autophagy is activated in response to γ irradiation and various anticancer therapies, particularly in apoptosis-deficient cells.[5,6] Several molecular and cell-signaling pathways have been implicated in regulating autophagy, such as the BECN1, AMP-activated protein kinase (AMPK), mitogen-activated kinase (MAPK), and phosphatidylinositol 3-kinase/AKT/mammalian target of rapamycin (PI3K−AKT−mTOR) pathways.[7,8] However, the detailed mechanisms of the autophagic cell-signaling pathway are still poorly understood.

α-Mangostin (5-hydroxy-2-methyl-1,4-naphthoquinone; Figure 1A) is a quinonoid constituent isolated from mangosteen, a tropical fruit native to southeast Asia. It has been shown to possess antioxidants and stabilize digestion.[9,10] α-Mangostin also has anticancer and antiproliferative properties in leukemia as well as prostate, breast, and colorectal cancers.[11−15] However, the effect of α-mangostin on brain cancer and the molecular

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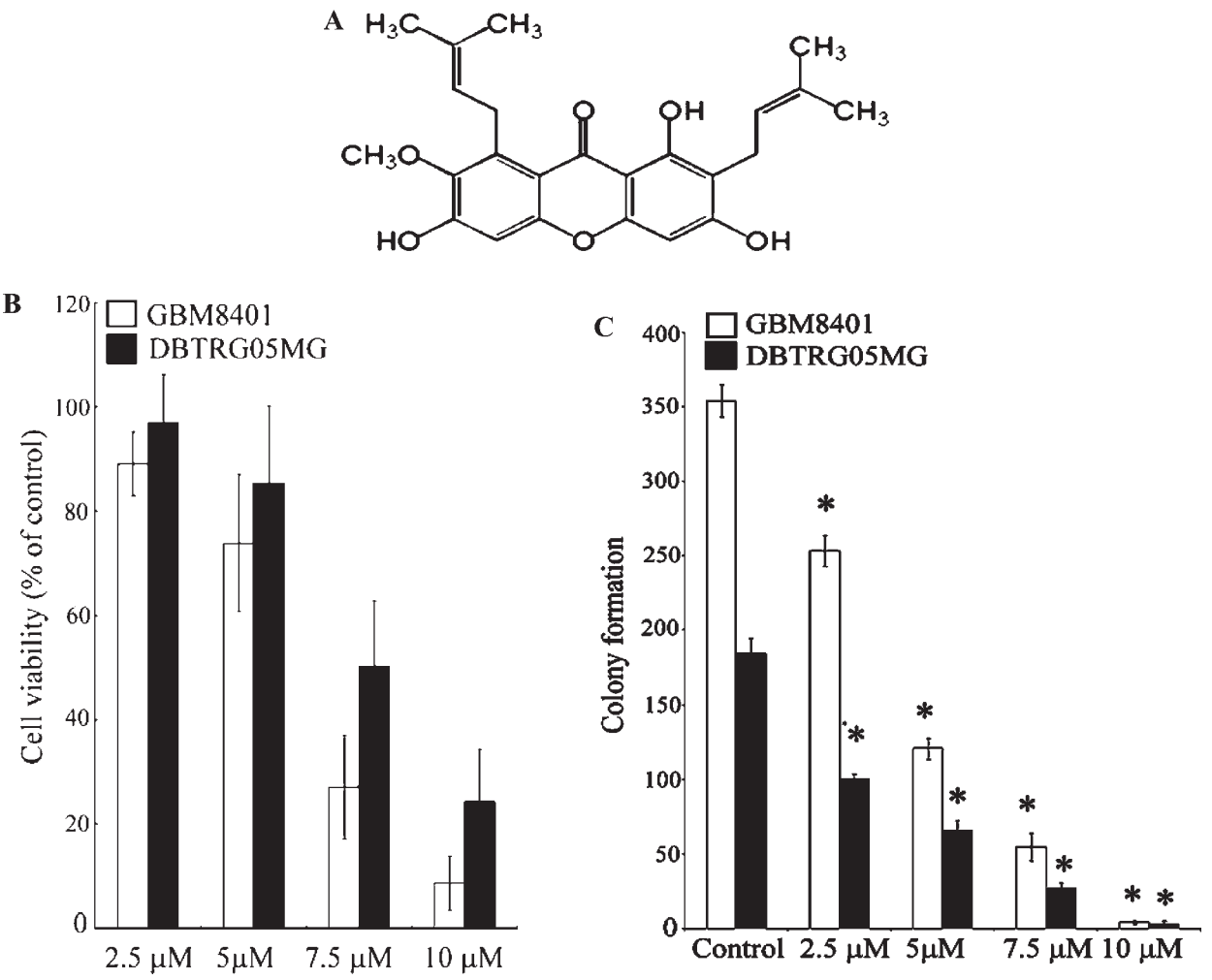

Figure 1. Effects of α-mangostin on cell viability and colony formation in GBM8401 and DBTRG-05MG cells. (A) Chemical structure of α-mangostin. (B) Effect of α-mangostin on cell viability in GBM8401 and DBTRG-05MG cells. (C) Influence of brain cancer cells on the number of colony-forming cells, as evaluated by the clonogenic assay. (A) Cell growth inhibition activity of α-mangostin was assessed by WST-1. For the colony-forming assay, the clonogenic assay was performed as described in the Materials and Methods. Each value is the mean ± SD of three determinations. Results shown are representative of three independent experiments. The asterisk indicates a significant difference between the control and α-mangostin-treated cells, (∗) $p < 0.05$.

mechanism of its effect have not yet been fully determined. In this study, we determined the effect of α-mangostin on cell growth inhibition using *in vitro* and *In Vivo* experimental models. We measured its effect on cell viability and autophagic cell death in two human brain cancer cell lines, GBM8401 and DBTRG-05MG. Furthermore, to establish the anticancer mechanism of α-mangostin, we determined the levels of autophagy-related molecules, which are strongly associated with the cell death signal transduction pathway and also affect the sensitivity of tumor cells to anticancer agents.

MATERIALS AND METHODS

Chemicals and Reagents. Fetal calf serum (FCS), RPMI 1640, penicillin G, streptomycin, and amphotericin B were obtained from Gibco BRL (Gaithersburg, MD). α-Mangostin, acridine orange, monodansylcadaverine (MDC), 3-methyladenine (3-MA), dimethyl sulfoxide (DMSO), ribonuclease (RNase), trypsin−ethylenediaminetetraacetic acid (EDTA), and propidium iodide (PI) were purchased from Sigma Chemical (St. Louis, MO). Liver kinase B1 (LKB1), phospho-LKB, AMP-activated protein kinase (AMPK), and phospho-AMPK antibodies were obtained from Cell Signaling Technology (Beverly, MA).

Cell Culture. The GBM8401 (BCRC 60163) and DBTRG-05MG (BCRC 60380) cell lines were obtained from the Bioresource Collection and Research Center (Hsinchu, Taiwan). GBM8401 cells were cultured in RPMI 1640 medium with 10% FCS. DBTRG-05MG cells were cultured in RPMI 1640 medium with 10 mg/L adenine, 1 mg/L adenosine triphosphate, 100 mg/L L-cytine, 5950 mg/L *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES), 15 mg/L hypoxanthine, 50 mg/L L-isoleucine, 50 mg/L L-proline, 100 mg/L sodium pyruvate, 1 mg/L thymidine, and 10% FCS.

Cell Viability and Clonogenic Assay. Cell viability was measured by the WST-1 assay. Cells were first plated in 96-well culture plates (1×10^4 cells/well). After 24 h of incubation, the cells were treated with vehicle alone (0.1% DMSO) or various concentrations of α-mangostin for 48 h. A total of 10 μL of WST-1 was then added to each well. An absorbance was measured on an enzyme-linked immunosorbent assay (ELISA) reader (Multiskan EX, Labsystems) at a wavelength of 450 nm. The percentage of inhibition was calculated using the following formula: percent inhibition = $[100 - (OD_t/OD_s) \times 100\%]$. OD_t and OD_s indicate the optical density of the test substances and the solvent control, respectively.

To determine the long-term effects of α-mangostin, cells were treated with α-mangostin at various concentrations for 1 h. After the cells were rinsed with fresh medium, they were allowed to grow and form colonies for 14 days and then were stained with crystal violet (0.4 g/L; Sigma).

Apoptosis Assay. Cells (1×10^6) were treated with vehicle alone (0.1% DMSO) or various concentrations of α-mangostin for 48 h and then were collected by centrifugation. Pellets were lysed with DNA lysis buffer (10 mM Tris at pH 7.5, 400 mM EDTA, and 1% Triton X-100) and then centrifuged. The supernatant obtained was incubated overnight with proteinase K (0.1 mg/mL) and then with RNase (0.2 mg/mL) for 2 h at 37 °C. After extraction with phenol/chloroform (1:1), the DNA was separated on a 2% agarose gel and visualized by ultraviolet (UV) after staining with ethidium bromide. Apoptotic cells were quantitatively assessed by the terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick end-labeling (TUNEL) method, which examines DNA-strand breaks during apoptosis. We used the BD ApoAlert DNA fragmentation assay kit.

Detection of Acidic Vesicular Organelles with Acridine Orange Staining. Autophagy is the process of sequestering cytoplasmic proteins into lytic components, which is characterized by the formation and promotion of acidic vesicular organelles (AVOs). To assess the occurrence of AVOs, we treated tumor cells with α-mangostin for 12 h and then stained them with acridine orange. Briefly, cells were incubated with acridine orange (1 μg/mL) for 15 min and then examined under a fluorescent microscope.[16,17]

Detection and Quantification of Autophagic Vacuoles with Monodansylcadaverine. Autophagic vacuoles were also detected with monodansylcadaverine (MDC) by incubating cells with MDC (50 μM) in phosphate-buffered saline (PBS) at 37 °C for 10 min. After incubation, cells were washed 4 times with PBS and immediately analyzed by fluorescent microscopy using an inverted microscope (Nikon Eclipse TE 300, Germany) equipped with a filter system (excitation wavelength, 380 nm; emission filter, 525 nm). To quantify MDC incorporation, at least 100 cells from each treatment group were examined by fluorescence microscopy and the percentage of MDC incorporation was calculated.[16,17]

EGFP-LC3 Plasmid Transfection. To study the formation of autophagic vacuoles, the localization of LC3, a specific marker of autophagosomes, was monitored. LC3 was expressed with an amino-terminal fusion of green fluorescent protein (EGFP), using Lipofectamine (Invitrogen) for GBM8401 and DBTRG-05MG cells. The plasmid encoding EGFP-LC3 was provided by Professor Tamotsu Yoshimori (Department of Cellular Regulation, Research Institute for Microbial Diseases, Osaka University, Osaka, Japan).

Electron Microscopy. Cells or tumor sections were directly fixed with 1% glutaraldehyde and post-fixed with 2% osmium tetroxide. The cell pellets or sections were embedded in Epon resin. Representative areas were chosen for ultra-thin sectioning and viewed with a JEM 1010 transmission electron microscope (JEOL, Peabody, MA).

Immunoblot Assay. Cells were treated with various concentrations of α-mangostin for the indicated times. For immunoblot, cells were lysed on ice for 15 min in radioimmunoprecipitation assay (RIPA) buffer. The cell lysate was then centrifuged at 12000*g* for 15 min, and the supernatant fraction was collected for immunoblot. Equivalent amounts of protein were resolved by sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE) (6−12%) and transferred to polyvinylidene difluoride (PVDF) membranes. After the membrane was blocked for 1 h in 5% nonfat dry milk in Tris-buffered saline, it was incubated with primary antibody for 1−16 h. The membrane was then treated with the appropriate peroxidase-conjugated secondary antibody, and the immunoreactive proteins were detected using an enhanced chemiluminescence kit (Amersham Biosciences) according to the instructions of the manufacturer.

AMPK and Beclin-1 Knockdown. RNAi reagents were obtained from the National RNAi Core Facility located at the Institute of Molecular Biology/Genomic Research Center, Academia Sinica, Taiwan. Cells were transfected with the shRNA plasmids (clones TRCN0000033549 for beclin-1 and clones TRCN0000000861 for AMPK) and selected by puromycin. The expression of AMPK and beclin-1 was determined by Q-PCR or immunoblot, and the stable clones with the highest knockdown efficiency were used for this study.

***In Vivo* Tumor Xenograft Study.** Male nude mice [6 weeks old; BALB/cA-*nu* (*nu*/*nu*)] were purchased from the National Science Council Animal Center (Taipei, Taiwan) and maintained in pathogen-free conditions. GBM8401 cells were injected subcutaneously into the flanks of these nude mice (5×10^6 cells in 200 μL), and tumors were allowed to develop for 15 days until they reached 200 mm^3, at which point treatment was initiated. A total of 20 mice were randomly divided into two groups. The mice in the α-mangostin-treated group were intraperitoneally injected daily with α-mangostin in a clear solution containing 25% polyethylene glycol (PEG) (2 mg/kg of body weight) in a volume of 0.2 mL. The control group was treated with an equal volume of vehicle. After transplantation, the tumor size was measured using calipers and the tumor volume was estimated according to the following formula: tumor volume (mm^3) = $L \times W^2/2$, where L is the length and W is the width. Tumor-bearing mice were sacrificed after 28 days. Xenograft tumors, as well as other vital organs of the treated and control mice, were harvested and fixed in 4% formalin, embedded in paraffin, and then cut into 4 μm sections for histologic study.

Statistical Analysis. Data are expressed as the mean ± standard deviation (SD). Statistical comparisons of the results were made using analysis of variance (ANOVA). Significant differences ($p < 0.05$) between the mean of the control and α-mangostin-treated cells were analyzed by Dunnett's test.

RESULTS

α-Mangostin Decreases Cell Viability and Clonogenic Survival in GBM8401 and DBTRG-05MG Cells. To investigate the potential of α-mangostin to cause cell death of cancer cells, we first examined the effect of α-mangostin on cell viability and clonogenic survival in GBM8401 and DBTRG-05MG cells. As shown in Figure 1B, α-mangostin decreased cell viability in both cancer cell lines in a concentration-dependent manner. The IC_{50} values of α-mangostin were 6.4 μM for GBM8401 and 7.3 μM for DBTRG-05MG.

Additional experiments were performed to determine the antitumor effects of α-mangostin inhibition by analyzing *in vitro* clonogenic assays. *In vitro* clonogenic assays correlated well with *In Vivo* assays of tumorigenicity in nude mice.[18] Figure 1C shows the effects of α-mangostin on the relative clonogenicity of the control and the α-mangostin-treated GBM8401 and DBTRG-05MG cells. Clonogenicity of both cancer lines was completely inhibited upon exposure to α-mangostin.

α-Mangostin Does Not Induce Apoptosis in GBM8401 and DBTRG-05MG Cells. α-Mangostin has been reported to induce apoptosis in human cancer cell lines.[13−15] We investigated whether α-mangostin could also induce apoptosis in brain cancer. TUNEL results revealed that α-mangostin did not induce apoptotic cell death in GBM8401 or DBTRG-05MG cells after 48 h of treatment (Figure 2A). In addition, α-mangostin treatment at different concentrations also failed to induce "DNA ladder" fragmentation, a typical marker of apoptosis that is detectable by agarose gel electrophoresis at 48 h in GBM8401 and DBTRG-05MG cells (Figure 2B). Moreover, α-mangostin did not increase caspase-3, caspase-8, or caspase-9 activity

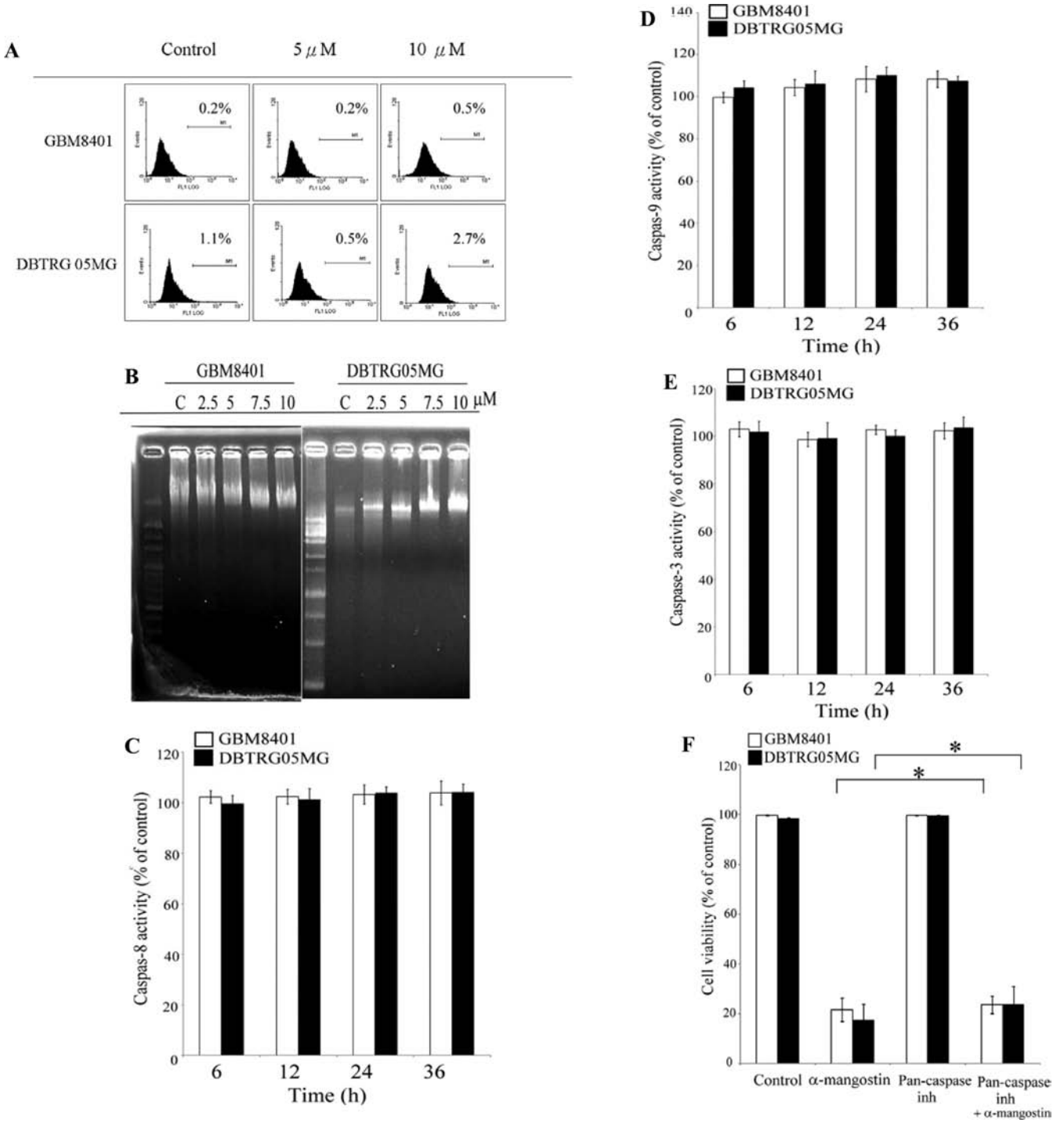

Figure 2. Effect of α-mangostin on the induction of apoptosis in brain cancer cells. α-Mangostin did not induce apoptosis in GBM8401 and DBTRG-05MG cells, as determined by (A) TUNEL staining and (B) agarose gel electrophoresis. The effect of α-mangostin on (C) caspase-8, (D) caspase-9, and (E) caspase-3. (F) Effect of a pan-caspase inhibitor on α-mangostin-mediated cytotoxicity. Cells were treated with various concentrations of α-mangostin for the indicated times and then stained using the ApoAlert DNA fragmentation assay kit. TUNEL-positive cells were examined by flow cytometry. DNA fragmentation was assessed by agarose gel electrophoresis. Caspase activities were assessed using caspase-8, caspase-9, and caspase-3 activity assay kits. (F) Cells were treated with z-VAD-FMK (20 μM) for 1 h. Then, 10 μM α-mangostin was added, and cells were incubated for 48 h. Cell viability was assessed by WST-1. Results shown are representative of three independent experiments.

in either GBM8401 or DBTRG-05MG cells (panels C−E of Figure 2).

To further determine whether apoptosis contributed to cellular toxicity in α-mangostin-treated brain cancer cells, we used a pan-caspase inhibitor to block caspase activity in both cancer cell lines and then determined whether the cell viability decreased. Blocking caspase activation did not decrease α-mangostin-mediated cytotoxicity in either cancer cell line, suggesting that α-mangostin does not induce brain cancer cells to undergo apoptosis (Figure 2F).

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α-Mangostin Induces Autophagy in GBM8401 and DBTRG-05MG Cells. Growing evidence indicates that non-apoptotic programmed cell death is principally attributable to autophagy (type-II programmed cell death).[3] The majority of α-mangostin-treated brain cancer cells do not display features typical of apoptosis. We next assessed whether α-mangostin induces autophagy in brain cancer. As shown in Figure 3A, α-mangostin treatment resulted in the appearance of AVO when cells were stained with acridine orange after a 12 h treatment. Because MDC accumulates in mature autophagic vacuoles, such as autophagolysosomes, but not in the early endosome compartment, MDC staining can be used to detect autophagic vacuoles. As shown in Figure 3A, treatment of cells with α-mangostin increased accumulation of MDC as compared to the control. These results corroborate with the observation that α-mangostin treatment induces autophagy in both cancer cell lines.

We also tested whether autophagy occurs in α-mangostin-treated cells using transmission electron microscopy (TEM). The results of TEM showed that, in most of the cells, the nuclei maintained their integrity and displayed dispersed chromatin, which is not consistent with the characteristics of cells undergoing apoptosis. Normal GBM8401 and DBTRG-05MG cells had numerous membrane-bound vesicles, often containing organelles and other cellular fragments (Figure 3B). In contrast, exposure of cells to 5 or 10 μM α-mangostin resulted in the appearance of autophagocytic vacuoles after a 12 h treatment. These autophagocytic vacuoles contained extensively degraded organelles (Figure 3B).

To determine the accumulation of autophagic vacuoles, we quantified MDC incorporation. At least 100 cells from each treatment group were examined using fluorescence microscopy, and the percentage of MDC incorporation was calculated. As shown in Figure 3C, we demonstrated that α-mangostin induces autophagy of GBM8401 and DBTRG-05MG cells in a dose-dependent manner. In addition, a 12 h exposure to 3-MA decreased the strength of blue fluorescence from 77.1 to 20.8% and from 75.7 to 20.6% in GBM8401 and DBTRG-05MG cells, respectively.

To examine alternative mechanisms of autophagy, we studied the effect of α-mangostin on the localization of LC3, an autophagy marker. During autophagy, cytosolic LC3I is cleaved to form the membrane-associated LC3II, which is involved in the formation of autophagosomes. In GBM8401 cells transfected with a plasmid encoding EGFP-LC3, α-mangostin exposure resulted in the redistribution of EGFP-LC3 into vesicular structures, as visualized by fluorescence microscopy, which is indicative of autophagy (Figure 3D).

To determine whether the cytotoxicity seen after brain cancer cells were treated with α-mangostin is caused by autophagy, siRNAs were used to knock down beclin-1 in GBM8401 and DBTRG-05MG cells. The reduction of beclin-1 (Figure 3E) resulted in the resistance to α-mangostin-mediated cytotoxicity in GBM8401 and DBTRG-05MG cells (Figure 3F). Moreover, an inhibitor of autophagosome/lysosome fusion,[19] bafilomycin, also decreased α-mangostin-mediated cytotoxicity (Figure 3G), suggesting that autophagy is the major type of cell death in α-mangostin-treated cancer cells.

α-Mangostin Increases LKB/AMPK Activation and Decreases mTORC1 Activity. Several cell signaling pathways have been reported to regulate autophagy;[3,4] consequently, we assessed the effect of α-mangostin on the ERK1/2, PI3K/AKT, and LKB/AMPK signaling pathways. As shown in Figure 4A, α-mangostin did not affect the expression of total PI3Kp85, PI3Kp110, AKT, PTEN, ERK1/2, LKB, and AMPK or the phosphorylation of either AKT or ERK1/2. In contrast, α-mangostin increased the phosphorylation of LKB and its downstream target AMPK.

Because AMPK has been reported to inhibit mTORC1 activity by increasing raptor phosphorylation,[7,20] we assessed the effect of α-mangostin on mTORC1 phosphorylation and its downstream targets. As shown in Figure 4B, treatment with α-mangostin decreased the phosphorylation of two substrates of mTORC1, p70S6K and 4E-BP1, revealing a potent inhibitory effect of α-mangostin on mTORC1 activity. α-Mangostin also increases phosphorylation of raptor (the regulatory associated protein of mTORC1) at Ser792. Furthermore, the interaction of raptor with 14−3−3γ also increased after α-mangostin exposure (Figure 4C).

Role of AMPK in α-Mangostin-Mediated mTORC1 Inhibition and Autophagy. To confirm the central role of AMPK in α-mangostin-induced autophagy and mTORC1 inhibition, we established stable GBM8401 and DBTRG-05MG colonies lacking AMPK (Figure 5A). AMPK-knockdown cells were treated with α-mangostin, and the induction of autophagy was assayed. Cells deficient in AMPK were more resistant to α-mangostin-triggered autophagy than cells transfected with the control plasmid (Figure 5B). The selective genetic inhibition of AMPK terminated α-mangostin-mediated raptor phosphorylation (Figure 5C). In addition, AMPK inhibition also prevented the interaction of raptor with 14−3−3γ in α-mangostin-treated GBM8401 and DBTRG-05MG cells (Figure 5D).

The role of AMPK in α-mangostin-mediated autophagy was further confirmed using the chemical AMPK inhibitor, compound C. Similar to genetic inhibition, treatment with compound C decreased α-mangostin-induced autophagy in both GBM8401 and DBTRG-05MG cells (Figure 6A). Compound C also prevented α-mangostin-induced raptor phosphorylation and the association of raptor/14−3−3γ (panels B and C of Figure 6). Therefore, these data suggest that α-mangostin induces autophagic cell death in brain cancer cells through the activation of the AMPK pathway and consequent inactivation of mTORC1.

α-Mangostin Inhibits Tumor Growth in Nude Mice. To determine whether α-mangostin inhibits tumor growth *In Vivo*, GBM8401 cells were injected subcutaneously into both flanks of the nude mice. Tumor growth inhibition was most evident in mice treated with α-mangostin at 2 mg kg^{-1} day^{-1}, where an approximately 50% reduction in tumor size was observed, as compared to mice treated with vehicle (Figure 7A). No signs of toxicity, as determined by parallel monitoring of body weight and tissue sections of lungs, liver, and kidneys, were observed in α-mangostin-treated mice (Figure 7B). Furthermore, TEM showed an increase of autophagocytic vacuoles in the tumors of the α-mangostin-treated mice when compared to the tumors of vehicle-treated mice (Figure 7C).

To determine the mechanism of the inhibition of tumor growth of α-mangostin *In Vivo*, we extracted proteins from the tumors to determine AMPK and raptor phosphorylation. As shown in Figure 7D, we observed an increase in phosphorylation of AMPK in the tumors of the α-mangostin-treated mice, as compared to the tumors from vehicle-treated mice. In addition, the phosphorylation of raptor was increased in the tumors from the α-mangostin-treated group when compared to tumors from vehicle-treated mice.

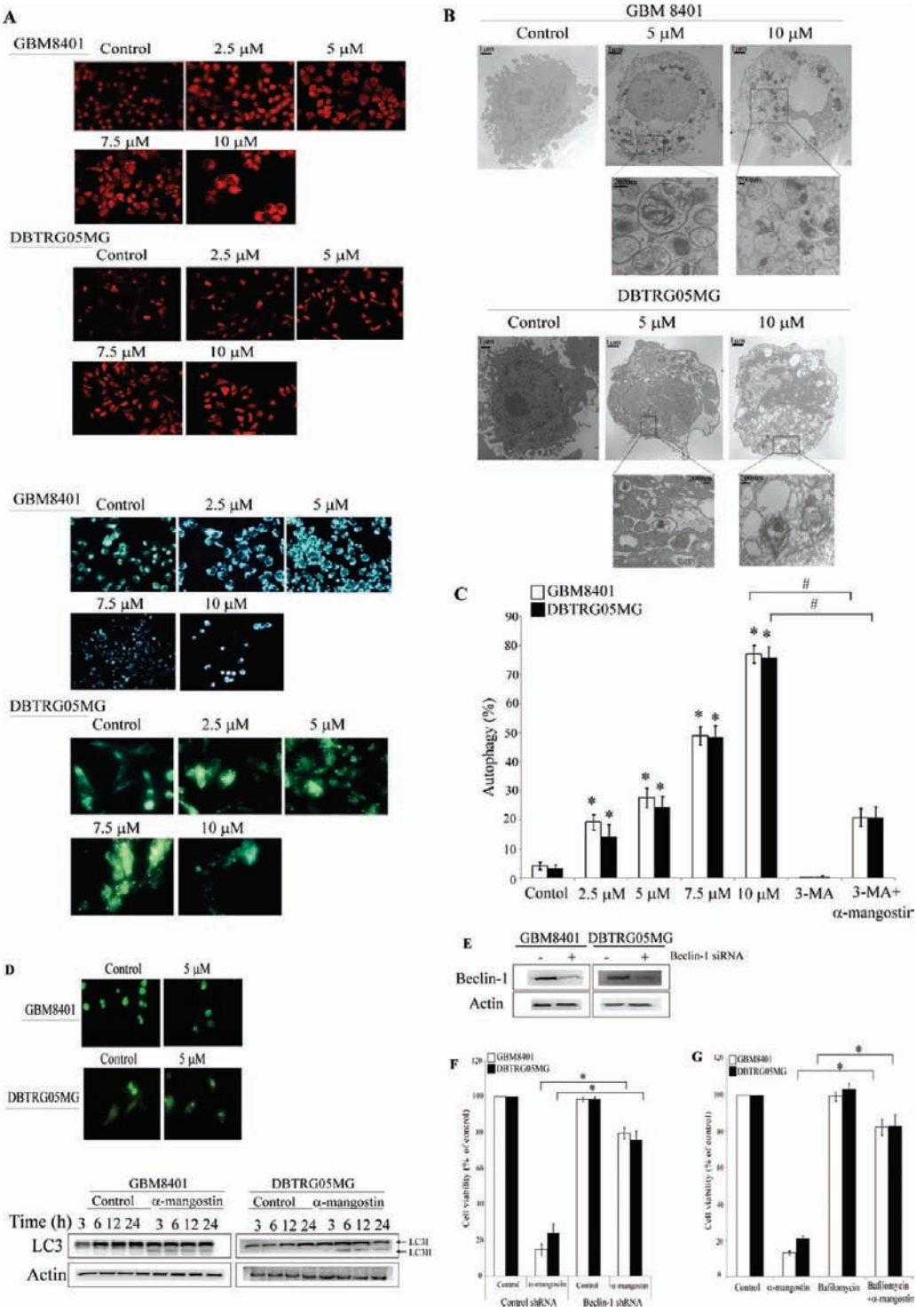

Figure 3. α-Mangostin induces autophagic cell death in two human brain cancer cell lines. (A) α-Mangostin-treated cells were stained with acridine orange and MDC. (B) Development of autophagocytic vacuoles was examined by transmission electron microscopy. Numerous autophagic and empty vacuoles were observed. (C) Quantification of MDC staining using fluorescence microscopy. (D) Aggregation of LC3 in α-mangostin-treated cells. (E) Inhibition of beclin-1 expression by siRNA transfection. (F) Effect of reducing beclin-1 siRNA on α-mangostin-mediated cytotoxicity. (G) Bafilomycin decreased α-mangostin-mediated cytotoxicity. Cells were treated with 5 or 10 μM α-mangostin for 12 h and then stained with acridine orange or MDC. After staining, cells were examined by fluorescence microscopy. EGFP-LC3 transfected cells were treated with α-mangostin for 12 h and then examined by fluorescence microscopy. The amount of LC3I and LC3II was assessed by immunoblot. For blocking experiments, cells were preincubated with 3-MA (5 mM) for 1 h before the addition of 10 μM α-mangostin for an additional 12 h. Results shown are representative of three independent experiments. The asterisk indicates a significant difference between the control and α-mangostin-treated cells or two test groups, (∗) $p < 0.05$.

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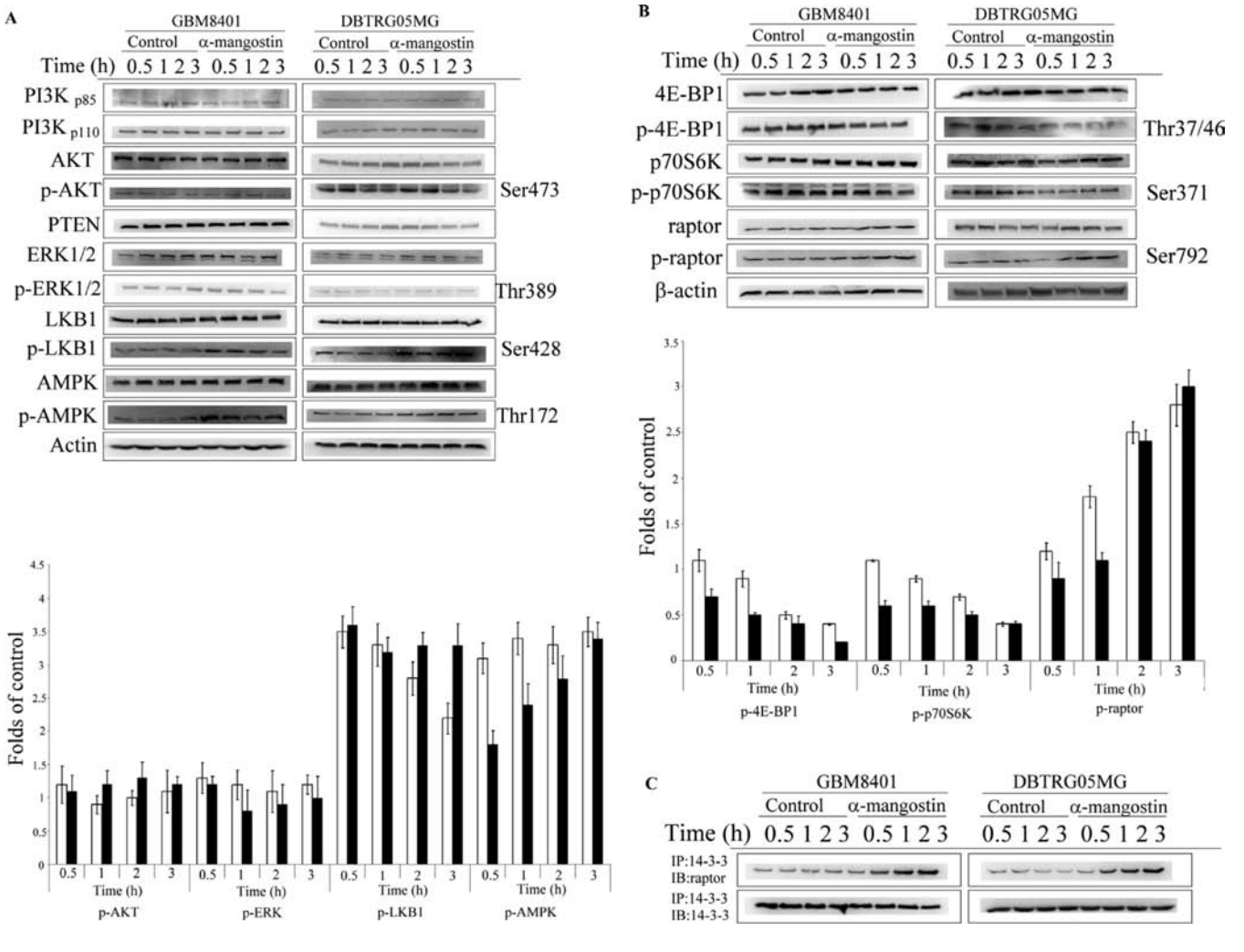

Figure 4. α-Mangostin increased the activation of the LKB and AMPK pathways. (A) Effect of α-mangostin on cell signaling involved in the regulation of autophagy. (B) α-Mangostin increased raptor phosphorylation and decreased mTORC1 activity. (C) α-Mangostin increased the interaction of raptor and 14−3−3γ. (A) Cells were treated with 10 μM α-mangostin at different times. The control cells received an equal volume of DMSO as the experimental cells. Cell lysates were prepared, and immunoblot was performed using antibodies against signaling proteins. The interaction of raptor with 14−3−3γ was determined by immunoprecipitation. Results shown are representative of three independent experiments.

DISCUSSION

Brain cancer is the most lethal human neoplasm.[1,2] In our study, we found that α-mangostin effectively inhibits tumor cell growth *in vitro*, concomitant with the induction of autophagic cell death, and furthermore, inhibits tumor cell growth in nude mice.

In the present study, we show that α-mangostin induces autophagic cell death but not apoptosis in GBM8401 and DBTRG-05MG cells. Some types of cancer cells exhibit changes in autophagy after treatment with chemotherapeutic drugs.[21,22] Autophagy begins with the sequestering of cytosolic components, including intracellular organelles within double-membrane structures. These vacuoles, called autophagosomes, undergo acidification after maturation. Once the autophagosomes fuse with lysosomes, their material is digested by lysosomal hydrolases.[3,4] Our results show the formations of AVOs in both GBM8401 and DBTRG-05MG cells, which were visualized by acridine orange and MDC staining after exposure to α-mangostin. In contrast, the typical characteristics of apoptosis, DNA fragmentation, and TUNEL staining are minimal or not observable in α-mangostin-treated GBM8401 and DBTRG-05MG cells. It is still unclear whether autophagy suppresses tumorigenesis or serves as a protective response for cancer cells in unfavorable conditions. Several studies have reported that autophagy is triggered in response to various anticancer agents.[21,22] Here, we show that α-mangostin-induced autophagy and autophagic cell death were blocked by 3-MA, Baf A1, and shRNA knockdown of beclin-1, all treatments known to inihibit canonical autophagy.

LKB/AMPK signaling is an important pathway in cancer cells and plays a variety of physiologic roles in cell growth, survival, migration, and cell-cycle regulation.[23,24] The serine/threonine kinase, LKB1, is the major regulator of AMPK through phosphorylation of AMPK at the activation loop, specifically at threonine 172 of the α subunit.[23,24] Recent studies have indicated that the activation of the LKB/AMPK pathway is associated with autophagy in cancer cells.[23−25] Our results show that α-mangostin treatment increased the phosphorylation of LKB, which was followed by an increase of AMPK activation. In addition, selective knockdown of AMPK expression by AMPK shRNA decreased α-mangostin-induced autophagy. These data are further supported by data from the chemical AMPK inhibitor,

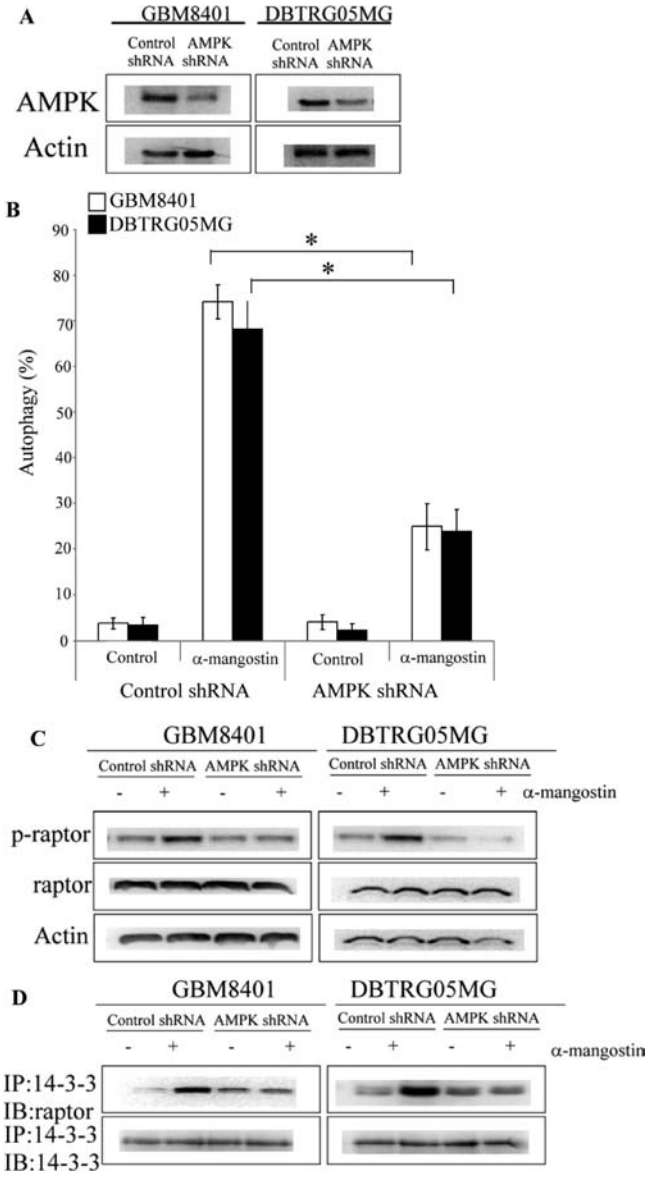

Figure 5. Role of AMPK in α-mangostin-mediated autophagic cell death. (A) Genetic suppression of AMPK by AMPK siRNA transfection. (B) Quantification of MDC staining using fluorescence microscopy. (C) Effect of AMPK inhibition on raptor phosphorylation. (D) AMPK siRNA decreased the interaction of raptor with 14−3−3γ. AMPK was knocked down in GBM8401 and DBTRG-05MG cells by transfection of shRNA targeting AMPK. Cells were treated with α-mangostin for 12 h for autophagic cell death or 3 h for raptor phosphorylation and the interaction of raptor/14−3−3γ. The degree of autophagy was assessed by MDC staining followed by fluorescence microscopy. Results shown are representative of three independent experiments. The asterisk indicates a significant difference between the control and α-mangostin-treated cells, (∗) $p < 0.05$.

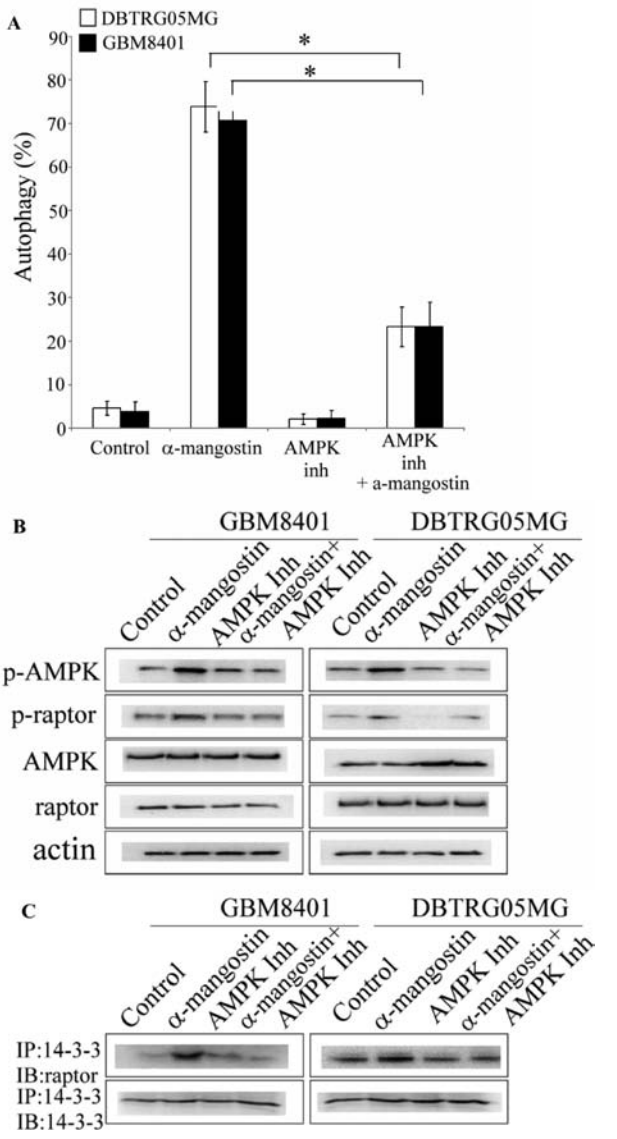

Figure 6. Chemical inhibition of AMPK decreases α-mangostin-mediated autophagic cell death. (A) Treatment with compound C inhibited α-mangostin-induced autophagy and decreased (B) α-mangostin-mediated phosphorylation of AMPK and raptor and (C) the interaction of raptor with 14−3−3γ protein. The degree of autophagy was assessed by MDC staining followed by fluorescence microscopy. Cells were pretreated with compound C for 1 h, and then 10 μM α-mangostin was added for 12 h for autophagic cell death, 0.5 h for AMPK expression, or 3 h for raptor phosphorylation and the interaction of raptor/14−3−3γ. The expression of various proteins was assessed by the immunoblot assay, and the interaction of raptor with 14−3−3γ was determined by immunoprecipitation. Results shown are representative of three independent experiments. The asterisk indicates a significant difference between the control and α-mangostin-treated cells, (∗) $p < 0.05$.

compound C, which decreased the number of GBM8401 and DBTRG-05MG cells that underwent autophagic cell death after α-mangostin treatment. Together, these findings indicate that α-mangostin induces autophagic cell death through the LKB/AMPK pathway in human brain cancer cells.

The mTOR complex 1 (mTORC1) is an important regulator of proliferation, survival, metabolism, apoptosis, and autophagy.[26] The mTORC1 complex consists of the scaffolding protein regulatory-associated protein of mTOR (raptor), mammalian lethal with Sec13 protein 8 (mLST8), proline-rich Akt substrate 40 kDa (PRAS40), and DEP-domain-containing mTOR-interacting protein (Deptor).[26,27] The activity of mTORC1 is regulated by various factors, including PI3K/AKT and AMPK.[28−30] AMPK can phosphorylate raptor, which is then sequestered by 14−3−3γ, resulting in mTORC1 inhibition.[30]

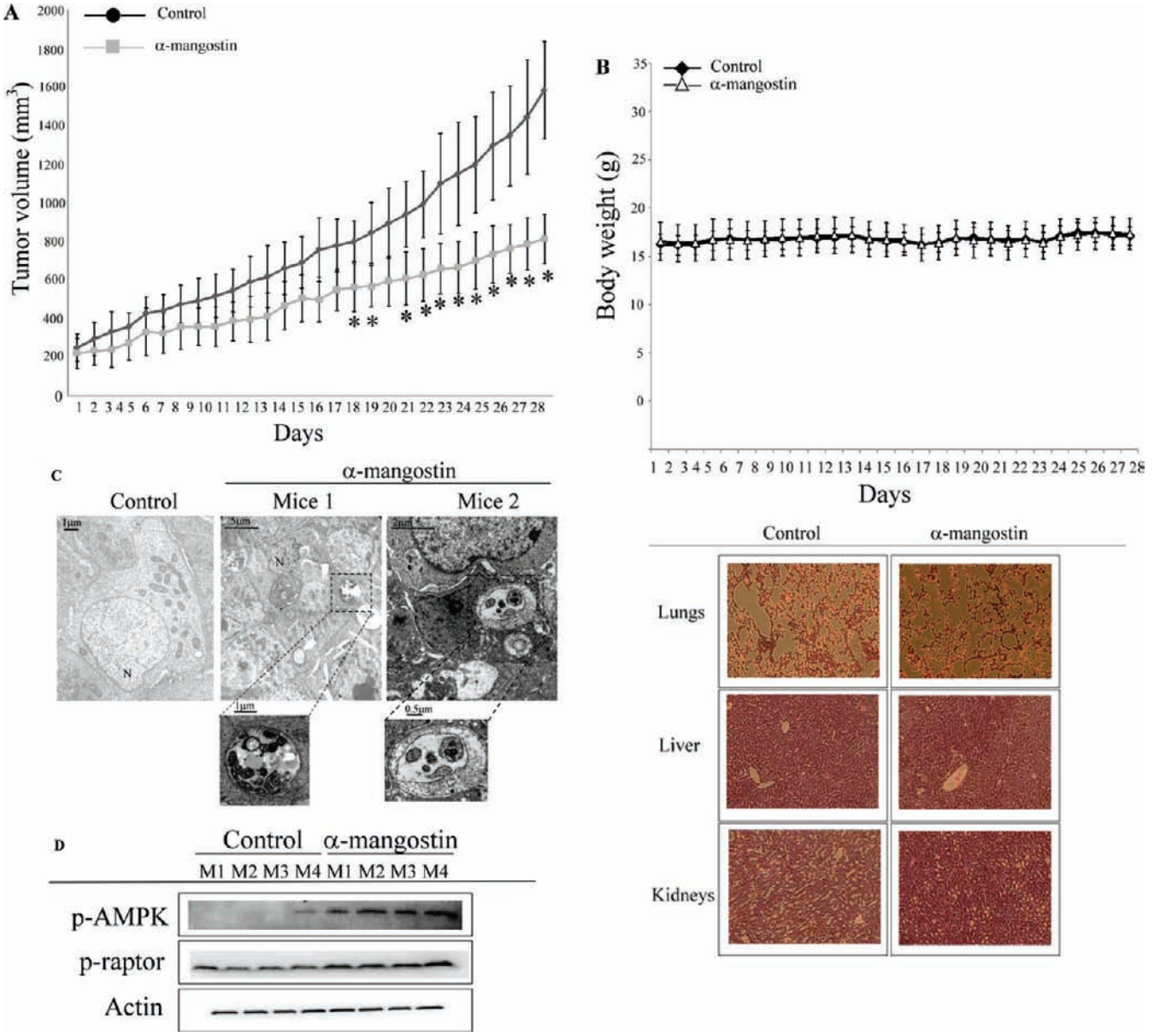

Figure 7. α-Mangostin inhibits growth of GBM8401 cells in nude mice. (A) Representative tumor-possessing nude mice and tumors from the control and α-mangostin-treated groups. (B) Mean of the tumor volume measured at the indicated number of days after implant. Tissue sections of the liver, lungs, and kidneys of α-mangostin-treated nude mice, as determined by haematoxylin and eosin (H&E) stain. (C) α-Mangostin induced autophagic cell death in the tumors of nude mice, as determined by TEM. Numerous autophagic and empty vacuoles were observed. (D) α-Mangostin increased the phosphorylation of AMPK and raptor signaling protein in GBM8401 xenograft. Animals bearing pre-established tumors (n = 10 per group) were dosed daily for 28 days with intraperitoneal injections of α-mangostin (2 mg kg^{-1} day^{-1}) or vehicle. During the 28 day treatment period, tumor volumes were estimated using measurements taken with external calipers (mm^3). The levels of various proteins were assessed by immunoblot analysis. Each value is the mean ± SD of three determinations. The asterisk indicates a significant difference between the control and α-mangostin-treated cells, (*) $p < 0.05$.

In our study, we found that AMPK is involved in inactive phosphorylation of raptor and association of raptor with the inhibitor 14−3−3γ, resulting in mTORC1 inhibition. These effects, however, were halted in GBM8401 and DBTRG-05MG cells that were co-treated with an AMPK inhibitor. Moreover, selective knockdown of AMPK expression by siRNA-based inhibition decreased the effects of α-mangostin on raptor phosphorylation and raptor/14−3−3γ interaction, suggesting that the cooperation of AMPK with mTORC1 plays a crucial role in α-mangostin-induced cell death in human brain cancer cells.

In conclusion, this study demonstrates that (a) the brain cancer cell lines, GBM8401 and DBTRG-05MG, are highly sensitive to growth inhibition by α-mangostin both *in vitro* and *In Vivo*, (b) reduced survival of brain cancer cells after exposure to α-mangostin is associated with autophagic cell death, and (c) α-mangostin-induced autophagic cell death in human brain cancer cells is mediated by the activation of the LKB/AMPK pathway. Finally, (d) it has been demonstrated that AMPK activation by α-mangostin could also inhibit mTORC1 activity by increasing raptor phosphorylation (Figure 8). These results provide a basic mechanism for the chemotherapeutic properties

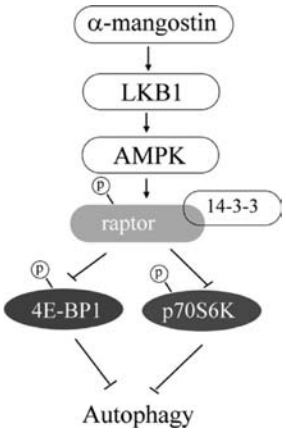

Figure 8. Model of action of α-mangostin. Proposed model for how α-mangostin affects various biochemical processes and events in GBM8401 and DBTRG-05MG cells and results in autophagic cell death is illustrated in this schematic diagram.

of α-mangostin in brain cancer cells. The overall effects of α-mangostin can contribute to the fight against brain cancer and might have future therapeutic applications.

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